



Eur päisches Pat ntamt
European Patent Office
Office européen des brevets

⑪ Publication number:

0 124 313
B1

⑫

EUROPEAN PATENT SPECIFICATION

⑮ Date of publication of patent specification: 02.08.89 ⑯ Int. Cl. 4: **C 07 K 1/00, C 12 P 21/02**
⑯ Application number: 84302577.6
⑯ Date of filing: 16.04.84

④ Process for the production of L-aspartyl-L-phenylalanine methyl ester or L-aspartyl-L-phenylalanine.

⑩ Priority: 28.04.83 JP 75558/83
28.04.83 JP 75559/83

⑩ Date of publication of application:
07.11.84 Bulletin 84/45

⑩ Publication of the grant of the patent:
02.08.89 Bulletin 89/31

⑩ Designated Contracting States:
CH DE FR GB IT LI NL

⑩ References cited:
EP-A-0 074 095
DD-A- 141 323
GB-A-2 092 161

FERMENTATION TECHNOLOGY TODAY,
PROCEEDINGS OF THE 4TH INTERNATIONAL
FERMENTATION SYMPOSIUM, Kyoto, JP,
19th-25th March 1972, pages 279-282, Gyozo
Tani, JP; T. HATA et al.: "Yeast proteinase"
CHEMICAL ABSTRACTS, vol. 81, no. 9, 2nd
September 1974, page 309, abstract no.
48563k, Columbus, Ohio, US; & CZ - A - 151 964
(DROBNICA, RUDOVIT) 15-01-1974

⑩ Proprietor: AJINOMOTO CO., INC.
5-8, Kyobashi 1-chome, Chuo-ku
Tokyo 104 (JP)

⑩ Inventor: Yokozeki, Kenzo
1523-4, Shinsaku Takatsu-ku
Kawasaki-shi Kanagawa-ken (JP)
Inventor: Kubota, Koji
6-4-6, Nagao Tama-ku
Kawasaki-shi Kanagawa-ken (JP)

⑩ Representative: Armitage, Ian Michael et al
MEWBURN ELLIS & CO. 2/3 Cursitor Street
London EC4A 1BQ (GB)

CHEMICAL ABSTRACTS, vol. 78, no. 15, 16th
April 1973, page 318, abstract no. 95705f,
Columbus, Ohio, US; H.E. MUELLER et al.:
"Proteolytic activity of *Cryptococcus*
neoformans against human plasma proteins",
& MED. MICROBIOL. IMMUNOL. 1972, 158(2),
129-34

CHEMICAL ABSTRACTS, vol. 85, no. 19, 18th
November 1976, page 239, abstract no.
139540z, Columbus, Ohio, US; M. GUEGUEN et
al.: "Characteristics of the proteolytic system
of *Geotrichum candidum*", & LAIT 1976,
56(557), 439-48

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European patent convention).

EP 0 124 313 B1

EP 0 124 313 B1

Description

BACKGROUND OF THE INVENTION

Field of the Invention

5 This invention relates to a process for producing L-aspartyl-L-phenylalanine methyl ester (abbreviated as APM hereinafter) or L-aspartyl-L-phenylalanine (abbreviated as AP hereinafter).

Description of the Prior Art

APM is a peptide which is noted as a sweetener in recent years.
10 It is well-known that the processes for the production of APM or AP include a chemical synthesizing process and an enzymatic synthesizing process.

The chemical synthesizing process for the production of APM comprises condensing N-protected L-aspartic acid anhydride and L-phenylalanine methyl ester (abbreviated as PM hereinafter) to obtain N-protected APM and then removing the protective group afterwards. The enzymatic synthesizing process 15 comprises exerting the effect of a protein-decomposing enzyme on N-protected L-aspartic acid and PM to obtain N-protected APM or the PM adduct of N-protected APM and then removing the protective group to form AP. However, both processes require the complicated steps of introducing the protective groups and removing the same.

There is also known a process for producing APM without using the protective groups (Japanese 20 Patent Kokai No. 43793/1983, "Digests of the Publications at the Annual Meeting of the Agricultural Chemical Society of Japan" in 1983, p. 42) which is a microbiological synthetic process using one of Pseudomonas, Torulopsis, Rhodotorula, and Sporobolomyces, but this is not always suitable for the industrial production of APM because of the extremely low yields.

25 SUMMARY OF THE INVENTION

The inventors of this application have eagerly sought a more effective process than the conventional one and have found that the employment of microorganisms brings about the direct and effective formation of APM from L-aspartic acid and PM, or AP from L-aspartic acid and L-phenylalanine (abbreviated as P hereinafter).

30 Accordingly, this invention is directed to a process for the production of APM or AP, characterized by exerting the action of a microorganism chosen from one of the following groups: Arthrobacter, Cellulomonas, Brevibacterium, Corynebacterium, Candida, Cryptococcus, Escherichia, Flavobacterium, Geotrichum, Micrococcus, Pachysolen, Saccharomyces, Trichosporon, Xanthomonas, Kluyveromyces, and Endomyces, and which has the ability to form APM or AP by the condensation of L-aspartic acid and PM or P to produce APM or AP.

35 The process for converting L-aspartic acid and PM to APM or L-aspartic acid and P to AP by conducting the condensation in an aqueous medium utilizing the action of microorganisms having the ability to form APM or AP by the condensation of L-aspartic acid and PM or P can easily be carried out only by contacting L-aspartic acid and PM or P with the microorganism cells, culture solutions, treated cells or active fractions 40 of the above-mentioned microorganisms.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following are examples of the microorganisms which have the ability to change L-aspartic acid and PM to APM or L-aspartic acid and P to AP by the condensation in this invention:

45	Corynebacterium sp.	ATCC 21251
	Corynebacterium xerosis	ATCC 373
	Candida intermedia	FERM-BP 508
	Cryptococcus neoformans	IFO 4289
50	Escherichia coli	FERM-BP 477
	Flavobacterium sewanens	FERM-BP 476
	Geotrichum candidum	IFO 4599
	Micrococcus luteus	ATCC 4698
	Pachysolen tannophilus	IFO 1007
55	Trichosporon capitatum	IFO 1197
	Xanthomonas campestris	FERM-BP 507
	Kluyveromyces thermotolerans	IFO 0662
	Endomyces ovotencis	IFO 1201
	Saccharomyces cerevisiae	IFO 2003
60	Arthrobacter citreus	ATCC 11624
	Cellulomonas flavigena	ATCC 8183
	Brevibacterium linens	ATCC 8377

65 The cells of these microorganisms can be obtained by using ordinary culture media. Further, L-aspartic acid and PM or P may be added at the beginning or in the process of cultivation.

EP 0 124 313 B1

The culture media to be used for the microorganisms of this invention are ordinary ones containing usual carbon and nitrogen sources and inorganic ions in addition to L-aspartic acid and PM or P. Moreover, the addition of trace amounts of organic nutritive substances such as vitamins and amino acids often brings about desirable results.

5 The carbon sources suitable for use herein include carbohydrates such as glucose and sucrose, organic acids such as acetic acid, alcohols, and so on. The nitrogen sources suitable for use herein include ammonia gas, aqueous ammonia, ammonium salts, and so on. The inorganic ions are properly selected from magnesium ion, phosphoric acid ion, potassium ion, iron ion and so on when necessary.

10 The cultures are preferably conducted under aerobic conditions at pH 4—8, at suitable temperatures controlled within the range of 25—40°C, and for 1—10 days to obtain desirable results.

15 The microorganisms to be used in this invention include the whole culture solutions obtained after completion of the cultivation thereof, the microorganisms separated from the culture solutions, or washed microorganisms. Also, the microorganisms to be used in this invention may be freeze-dried, acetone-dried, contacted with toluene, surfactants, etc., treated with lysozyme, exposed to ultrasonic waves, mechanically ground. By microorganism is meant any of the above-mentioned treated or untreated microorganisms.

20 Furthermore, since it is an enzyme or a combination of enzymes contained in the microorganisms which bring about the condensation of the substrates to form the product, any fraction of the microorganisms which contains said enzyme or enzymes may be used in the practice of this invention. For example, an enzyme protein fraction, insolubilized materials resulting from the above-mentioned treatments, etc. may be used by the method of this invention. By enzyme-containing fraction of a microorganism is meant any such microorganism-derived, enzyme-containing material that is capable of bringing about the stated chemical reactions. One skilled in the art can easily determine whether any unknown microorganism or enzyme-containing fraction is within the scope of this invention by contacting the microorganism or fraction with the named starting materials and monitoring the formation of reaction products as described herein.

25 As aqueous media, there can be used those containing water, buffers, and organic solvents such as ethanol. Moreover, nutritive elements needed for the growth of microorganisms, anti-oxidants, surfactants, coenzymes, hydroxylamine and metallic ions, etc. can be added to the aqueous media if necessary.

30 When the cells of the above-mentioned microorganisms are grown in aqueous media and simultaneously brought into contact with L-aspartic acid and PM or P to exert the action thereon, the aqueous media should contain L-aspartic acid, PM or P, and also nutritive elements such as carbon sources, nitrogen sources, and inorganic ions, etc. needed for the growth of the microorganisms. Further, the addition of trace amounts of organic nutritive elements such as vitamins and amino acids often brings about desirable results.

35 The carbon sources suitable for use herein include carbohydrates such as glucose and sucrose, organic acids such as acetic acid, alcohols, and so on. The nitrogen sources suitable for use herein include ammonia gas, aqueous ammonia, ammonium salts, and so on. The organic ions are properly selected from magnesium ion, phosphoric acid ion, potassium ion, iron ion, and so on when necessary.

40 The microorganisms are preferably grown under aerobic conditions at pH 4—8, and at proper temperatures controlled within the range of 25—40°C to obtain desirable results.

45 Thus, L-aspartic acid and PM or P can be efficiently converted to APM or AP when incubated for 1—10 days.

50 When the whole culture solutions, culture cells or cell-treating materials of the above-mentioned microorganisms are brought directly into contact with L-aspartic acid and PM or P to exert the action thereon, the aqueous media prepared by dissolving or suspending L-aspartic acid, PM, or P and the culture solutions, microorganism culture cells, or microorganism cell-treating materials, and are controlled at proper temperatures of 10—70°C, kept at pH 4—8, and allowed to stand for a while or stirred, a great deal of APM or AP is produced and accumulated in the aqueous media after 5—100 hours.

55 The APM or AP thus produced can be separated and purified by the publicly-known process for separation. The APM or AP obtained was determined with an amino-acid analyzer.

60 The invention now being generally described, the same will be better understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting of the invention or any embodiment thereof.

65 Example 1

66 Into a 500 ml-flask was introduced 50 ml of a medium (pH 7.0) containing 2.0 g/dl of glucose, 0.5 g/dl or $(\text{NH}_4)_2\text{SO}_4$, 0.1 g/dl of KH_2PO_4 , 0.1 g/dl of K_2HPO_4 , 0.05 g/dl of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg/dl of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg/dl of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 1.0 g/dl of yeast extract, 0.5 g/dl of malt extract, and 4.0 g/dl of calcium carbonate, which was sterilized at 120°C for 15 minutes.

67 Each one of the thus prepared media was inoculated, using a platinum loop, with each of the microorganisms of Table 2, incubated in a bouillon-agar medium at 30°C for 24 hours, and cultured at 30°C for an additional 20 hours. The cells were harvested from this culture solution by centrifugation, washed once with the same amount of physiological saline as that of the culture solution and collected.

68 These cells of the microorganisms were added to Reaction Solution A shown in Table 1 to equal 5 g/dl

EP 0 124 313 B1

(final conditions, pH 5.4, 5 ml), and allowed to react at 37°C for 16 hours. The resulting APM was determined with an amino-acid analyzer to give the results in Table 2.

Table 1

Reaction Solution A		
	L-aspartic acid	10 g/dl
	L-phenylalanine methyl ester hydrochloride	20 g/dl

The above substrates are included in 0.1 M phosphoric acid buffer (final pH 5.4).

Table 2

Microorganisms	Reaction solution	APM formed (mg/dl)
Corynebacterium sp.	ATCC 21251	673
Corynebacterium xerosis	ATCC 373	252
Candida intermedia	FERM-BP 508	321
Cryptococcus neoformans	IFO 4289	142
Escherichia coli	FERM-BP 477	915
Flavobacterium sewanense	FERM-BP 476	820
Geotrichum candidum	IFO 4599	156
Micrococcus luteus	ATCC 4698	721
Pachysolen tannophilus	IFO 1007	121
Trichosporon capitatum	IFO 1197	127
Xanthomonas campestris	FERM-BP 507	328
Kluyveromyces thermotolerans	IFO 0662	116
Endomyces ovotencis	IFO 1201	364
Saccharomyces cerevisiae	IFO 2003	97
Arthrobacter citreus	ATCC 11624	910
Cellulomonas flavigena	ATCC 8183	620
Brevibacterium linens	ATCC 8377	820

Example 2

Into 100 ml of Reaction Solution A was introduced 5 g of Flavobacterium sewanense FERM-BP 476 grown and washed in a manner similar to Example 1, and the reaction was carried out at 37°C for 24 hours. The resulting reaction solution was spotted on a TLC plate for development in the form of a belt, and developed with a solvent system consisting of n-butanol:acetic acid:water = 2:1:1. Part of the product APM was taken out and extracted with distilled water. Then, the resulting reaction product was crystallized to obtain 560 mg of crystals. The obtained crystals were characterized as to optical rotation, melting point, and specific rotatory power, and the product obtained from Reaction Solution A was identical to an authentic APM specimen.

EP 0 124 313 B1

Example 3

5 Into the culture solution of *Escherichia coli* FERM-BP 477 grown at 30°C for 12 hours in the same medium used in Example 1 was poured under sterile conditions 10 ml of aqueous solution (adjusted to pH 5.4) containing 5 g/dl of L-aspartic acid and 10 g/dl of PM, and the cultivation was continued for 10 hours after the solution was adjusted under sterile conditions to pH 5.4. It was maintained at a pH of 5.4 by adjustments at intervals of 2 hours during incubation.

The resulting product in this culture solution was verified with an amino-acid analyzer and 320 mg/dl of APM was obtained.

Example 4

10 Into a 500 ml-flask was introduced 50 ml of a medium (pH 7.0) containing 2.0 g/dl of glucose, 0.5 g/dl of $(\text{NH}_4)_2\text{SO}_4$, 0.1 g/dl of KH_2PO_4 , 0.1 g/dl of K_2HPO_4 , 0.05 g/dl of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg/dl of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg/dl of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 1.0 g/dl of yeast extract, 0.5 g/dl of malt extract, and 4.0 g/dl of calcium carbonate, which was sterilized at 120°C for 15 minutes.

15 Each one of the thus prepared media was inoculated, using a platinum loop, with each of the microorganisms of Table 4, incubated in a bouillon-agar medium at 30°C for 24 hours, and cultured at 30°C for an additional 20 hours. The cells were harvested from this culture solution by centrifugation, washed once with the same amount of physiological saline as that of the culture solution and collected.

20 These cells of the microorganisms were added to Reaction Solution B shown in Table 3 to equal 5 g/dl (final conditions, pH 5.4, 5 ml), and allowed to react at 37°C for 16 hours. The resulting AP was determined with an amino acid analyzer to give the results in Table 4.

Table 3

Reaction Solution B	
L-aspartic acid	10 g/dl
L-phenylalanine	15 g/dl

30 The above substrates are included in 0.1 M phosphoric acid buffer (final pH 5.4).

35

40

45

50

55

60

65

EP 0 124 313 B1

Tabl 4

5	Microorganisms	Reaction solution
		APM formed (mg/dl)
10	<i>Corynebacterium</i> sp.	ATCC 21251 391
15	<i>Corynebacterium xerosis</i>	ATCC 373 103
20	<i>Candida intermedia</i>	FERM-BP 508 270
25	<i>Cryptococcus neoformans</i>	IFO 4289 101
30	<i>Escherichia coli</i>	FERM-BP 477 371
35	<i>Flavobacterium sewanense</i>	FERM-BP 476 435
40	<i>Geotrichum candidum</i>	IFO 4599 103
45	<i>Micrococcus luteus</i>	ATCC 4698 291
50	<i>Pachysolen tannophilus</i>	IFO 1007 93
55	<i>Trichosporon capitatum</i>	IFO 1197 101
60	<i>Torulopsis inconspicua</i>	IFO 0621 27
65	<i>Rhodotorula lactosa</i>	IFO 1424 29
70	<i>Xanthomonas campestris</i>	FERM-BP 507 108
75	<i>Kluyveromyces thermotolerans</i>	IFO 0662 54
80	<i>Endomyces ovetencis</i>	IFO 1201 121
85	<i>Saccharomyces cerevisiae</i>	IFO 2003 63
90	<i>Arthrobacter citreus</i>	ATCC 11624 380
95	<i>Cellulomonas flavigena</i>	ATCC 8183 280
100	<i>Brevibacterium linens</i>	ATCC 8377 340

Example 5

40 Into 100 ml of Reaction Solution B was introduced 5 g of *Flavobacterium sewanense* FERM-BP 476 grown and washed in a manner similar to Example 4, and the reaction was carried out at 37°C for 24 hours.

45 The resulting reaction solution was spotted on a TLC plate for development in the form of a belt and developed with a solvent system consisting of n-butanol:acetic acid:water = 2:1:1. Part of the produced AP was taken out and extracted with distilled water. Then the resulting reaction product was crystallized to obtain 250 mg of crystals. The obtained crystals were characterized as to optical rotation, melting point, and specific rotatory power, and the product was identical to an authentic AP specimen.

Example 6

50 Into the culture solution of *Escherichia coli* FERM-BP 477 grown at 30°C for 12 hours in the same medium as used in Example 4 was poured under sterile conditions 10 ml of aqueous solution (adjusted to pH 5.4) containing 5 g/dl of L-aspartic acid and 7 g/dl of L-phenylalanine, and the cultivation was further continued for 10 hours after the solution was adjusted under sterile conditions to pH 5.4. It was maintained at a pH of 5.4 by adjustments at intervals of 2 hours during incubation.

55 The resulting product in this culture solution was verified with an amino-acid analyzer and 180 mg/dl of AP was obtained.

Claims

60 1. A process for the production of a sweetening agent, characterised by:
 contacting in an aqueous medium L-aspartic acid and L-phenylalanine methyl ester with a microorganism or enzyme-containing fraction of said microorganism, said microorganism or fraction containing one or more enzymes capable of forming L-aspartyl-L-phenylalanine methyl ester by the condensation of L-aspartic acid and L-phenylalanine methyl ester; or
 65 contacting in an aqueous medium L-aspartic acid and L-phenylalanine with a microorganism or

EP 0 124 313 B1

enzyme-containing fraction of said microorganism, said microorganism or fraction containing one or more enzymes capable of forming L-aspartyl-L-phenylalanine by the condensation of L-aspartic acid and L-phenylalanine;

5 said microorganism belonging to a genus selected from Arthrobacter, Cellulomonas, Brevibacterium, Corynebacterium, Candida, Cryptococcus, Escherichia, Flavobacterium, Geotrichum, Micrococcus, Pachysolen, Saccharomyces, Trichosporon, Xanthomonas, Kluyveromyces, and Endomyces.

2. The process of Claim 1 wherein the microorganism is selected from:

10	Corynebacterium sp.	ATCC 21251
	Corynebacterium xerosis	ATCC 373
	Candida intermedia	FERM-BP 508
	Cryptococcus neoformans	IFO 4289
	Escherichia coli	FERM-BP 477
	Flavobacterium sewanens	FERM-BP 476
15	Geotrichum candidum	IFO 4599
	Micrococcus luteus	ATCC 4698
	Pachysolen tannophilus	IFO 1007
	Trichosporon capitatum	IFO 1197
	Xanthomonas campestris	FERM-BP 507
20	Kluyveromyces thermotolerans	IFO 0662
	Endomyces ovencis	IFO 1201
	Saccharomyces cerevisiae	IFO 2003
	Arthrobacter citreus	ATCC 11624
	Cellulomonas flavigena	ATCC 8183
25	Brevibacterium linens	ATCC 8377

3. The process of Claim 1 or Claim 2 wherein the microorganism is cultured under aerobic conditions at pH 4-8 and at a temperature of 25°C-40°C for a time period in the range of 1-10 days.

30 Patentansprüche

31 1. Verfahren zur Herstellung eines Süßmittels, dadurch gekennzeichnet, daß
in einem wässrigen Medium L-Asparaginsäure und L-Phenylalanin-methylester mit einem
Mikroorganismus oder einer enzymhaltigen Fraktion dieses Mikroorganismus in Kontakt gehalten werden,
wobei der Mikroorganismus oder die Fraktion ein oder mehrere Enzyme enthält, die zur Bildung von L-
35 Aspartyl-L-phenylalanin-methylester durch Kondensation von L-Asparaginsäure und L-Phenylalanin-
methylester befähigt sind, oder in einem wässrigen Medium L-Asparaginsäure und L-Phenylalanin mit
einem Mikroorganismus oder einer enzymhaltigen Fraktion dieses Mikroorganismus in Kontakt gehalten
werden, wobei dieser Mikroorganismus oder diese Fraktion ein oder mehrere Enzyme enthält, die zur
Bildung von L-Aspartyl-L-phenylalanin durch Kondensation von L-Asparaginsäure und L-Phenylalanin
40 befähigt sind, wobei der Mikroorganismus einem Genus angehört, der unter Arthrobacter, Cellulomonas,
Brevibacterium, Corynebacterium, Candida, Cryptococcus, Escherichia, Flavobacterium, Geotrichum,
Micrococcus, Pachysolen, Saccharomyces, Trichosporon, Xanthomonas, Kluyveromyces und Endomyces
ausgewählt ist.

45 2. Verfahren gemäß Anspruch 1, bei dem der Mikroorganismus ausgewählt ist unter:

45	Corynebacterium sp.	ATCC 21251
	Corynebacterium xerosis	ATCC 373
	Candida intermedia	FERM-BP 508
	Cryptococcus neoformans	IFO 4289
50	Escherichia coli	FERM-BP 477
	Flavobacterium sewanens	FERM-BP 476
	Geotrichum candidum	IFO 4599
	Micrococcus luteus	ATCC 4698
	Pachysolen tannophilus	IFO 1007
55	Trichosporon capitatum	IFO 1197
	Xanthomonas campestris	FERM-BP 507
	Kluyveromyces thermotolerans	IFO 0662
	Endomyces ovencis	IFO 1201
	Saccharomyces cerevisiae	IFO 2003
60	Arthrobacter citreus	ATCC 11624
	Cellulomonas flavigena	ATCC 8183
	Brevibacterium linens	ATCC 8377

65 3. Verfahren nach Anspruch 1 oder Anspruch 2, bei dem der Mikroorganismus unter aeroben
Bedingungen bei einem pH von 4 bis 8 und bei einer Temperatur von 25°C bis 40°C während einer Dauer im
Bereich von 1 bis 10 Tagen gezüchtet wird.